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Automated Continuous-Flow Colorimetric Determination of Glutathione Peroxidase with Dichloroindophenol[1]

Wayne C. Hawkes and Kimberly A. Craig

Western Human Nutrition Research Center, U.S. Department of Agriculture, Agricultural Research Service, Post Office Box 29997, Presidio of San Francisco, California 94129

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Automation of the glutathione peroxidase enzyme assay has been problematical. Although such methods have been reported, they do not give equivalent results to the standard manual assay, wherein glutathione oxidation is coupled to NADPH oxidation via glutathione reductase. We report here the development of a fully automated, continuous-flow, colorimetric method for glutathione peroxidase assays in which glutathione oxidation is monitored by its effect on the reaction of glutathione with the colorimetric reagent 2,6-dichloroindophenol. This method has a linear response to glutathione peroxidase over an 800-fold range of enzyme concentrations. Results of assays done by this method in erythrocyte and plasma samples correlate well with the standard manual coupled assay (r = 0.997 and 0.923, respectively), with no evidence of systematic errors. The assay works equally well with hydrogen peroxide or cumene hydroperoxide as substrate and shows the same selectivity toward glutathione *S*-transferases as the standard coupled assay. The within-day repeatability and the between-day reproducibility were estimated as 1.1 to 6.4% and 1.3 to 7.1% (relative standard deviation), respectively. This method is suitable for enzyme determinations in whole blood, erythrocytes, plasma, and serum from rats, rabbits, monkeys, and humans.

Glutathione peroxidase (glutathione:hydrogen peroxide oxidoreductase, EC 1.11.1.9) catalyzes the reduction of H_2O_2 and organic hydroperoxides by reduced GSH. The enzyme, first described by Mills in 1957 (1), is ubiquitous in animal tissues, but has not been reported in higher plants or bacteria. GSH-Px[2] is the major pathway in erythrocytes for the elimination of H_2O_2 (2). Selenium was shown to be an essential component of the erythrocyte enzyme in 1973 (3) and the form of selenium in the rat liver enzyme was identified as selenocysteine in 1978 (4).

The most widely accepted assay for GSH-Px is the method of Paglia and Valentine (5), in which the oxidation of GSH is coupled to NADPH oxidation by GSH reductase. Many modifications of the coupled assay have been published which vary in substrate concentrations, type of peroxide, presence of chelators and inhibitors, pH, and temperature (6–8). Because the selenium-containing enzyme cannot be simultaneously saturated with respect to both substrates (9), there has been no widespread agreement on a standard enzyme unit definition.

Other assay methods have been proposed for GSH-Px. The direct spectrophotometric measurement of GSH consumption at 237 nm (10) works well but suffers from a nonphysiological pH of 10.5 and a high nonenzymatic blank rate. The method based on indirect measurement of GSH consumption with DTNB (11,12) is based upon logarithmic units which exhibit very poor linear correlations with the coupled assay (13). Semiautomated procedures have been developed using DTNB to monitor GSH consumption continuously (14) or HPLC to separate and quantitate GSH and GSSG (15). An automated version of the coupled assay was developed on a centrifugal analyzer but showed a relatively poor correlation with the manual coupled procedure for assays of rat liver samples (r = 0.81), with significant constant and proportional errors (16). A recent report describes the adaptation of the coupled assay method to a random access chemistry analyzer (Technicon RA-1000) for assays of human plasma samples, but insufficient information was presented to evaluate its utility or cost (17).

In this paper, we describe a fully automated, continuous-flow, colorimetric assay for GSH-Px, using the reaction of DCIP with GSH to monitor the oxidation of GSH. The method exhibits a linear response to GSH-Px over an 800-fold range of concentrations. This assay

[1] Reference to a company or product name does not constitute endorsement by the U.S. Department of Agriculture.

[2] Abbreviations used: GSH-Px, glutathione peroxidase; DCIP, 2,6-dichloroindophenol; CHPO, cumene hydroperoxide; U, enzyme activity unit; mU, 10^{-3} enzyme activity units; DTNB, dithiobis-5,5′-(2-nitrobenzoic acid).

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correlates well with the manual coupled assay in blood from several species and has essentially the same characteristics as the coupled assay with respect to inhibition, substrate preference, and selectivity for the selenium-dependent form of the enzyme.

MATERIALS AND METHODS

Reagent preparation and stability. The 1.5 mM CHPO reagent, the 1.5 mM H_2O_2 reagent, and the 2.3 mM GSH/1 mM disodium–EDTA reagent (all from Sigma Chemical Co., St. Louis, MO) were prepared in cold distilled water. The reaction buffer was 40 mM NaCN/0.25 M Tris·HCl/0.1 mM disodium–EDTA, pH 7.8. The 0.68 mM DCIP reagent (Sigma) was prepared from a filtered stock solution of DCIP (80 absorbance units/ml at 624 nm, stable for 30 days at 4°C), by diluting to a concentration of 14 absorbance units/ml at 624 nm in cold distilled water. The above solutions were kept on ice and were discarded at the end of each day.

Preparation of samples and standards. A stock solution of bovine erythrocyte GSH-Px (Sigma No. G-6137) was prepared at a nominal concentration of 1 U/ml (Sigma units, Ref. (7)) in 50 mM Tris·HCl/0.1 mM EDTA/0.5% Triton X-100, pH 7.8, and stored in 1-ml aliquots at −70°C. The stock solution was calibrated with the manual coupled assay ((18), see below) to establish the relationship between their enzyme unit definition and ours. The stock solutions were thawed overnight at 4°C and working standards were prepared fresh each day. EDTA-treated human blood samples were obtained from Cocalico Biologicals (Reamstown, PA). Animal blood samples were excess samples from various previous studies. Blood samples (whole blood, packed erythrocytes, plasma, or serum—with various anticoagulants) were stored at −70°C and thawed overnight at 4°C. Thawed blood samples were treated for 2 min in an ultrasonic bath at 4°C to resuspend particulate matter and vortexed vigorously immediately before aliquots were withdrawn for assay. Seventy-five microliters of whole blood or erythrocytes or 0.1 ml of plasma or serum was diluted in 2.925 ml (40-fold) or in 1.9 ml (20-fold) of cold 50 mM Tris·HCl/0.1 mM EDTA/0.5% Triton X-100, pH 7.8, respectively. Blood samples showed no significant decrease in enzyme activity overnight at 4°C or for up to 2 h at room temperature in the automatic sampler.

Continuous-flow analytical system. The analytical system (Fig. 1) was constructed from standard Technicon Auto-Analyzer equipment (Technicon Industrial Systems, Tarrytown, NY). Transmission lines, mixing coils, and reaction coils were 2.4-mm-i.d. glass tubing. The three reactor coils were kept in insulated baths at ambient temperature. To accomodate the very different enzyme activities in whole blood/erythrocyte and plasma/serum samples, two sampling lines were included with a 10-fold difference in their flow rates. When plasma or serum samples were assayed, the 1.0 ml/min sample line was used for sampling and the 0.1 ml/min line was used to pump diluent (50 mM Tris·HCl/0.1 mM EDTA/0.5% Triton X-100, pH 7.8). When whole blood or erythrocytes were assayed, the 0.1 ml/min sample line was used for sampling and the 1.0 ml/min line was used to pump diluent. This approach permitted the sensitivity of the assay to be changed by a factor of 10, which increased the linear range by the same factor. The sampling rate was 30/h, with a sample-to-wash ratio of 1:1.

Data collection and processing. Data from the colorimeter were collected by a DP-1000 computerized data acquisition system (Labtronics, Guelph, Ontario, Canada). The raw data were expressed as net absorbance peak heights over baseline. Sample-to-sample carryover was measured at less than 0.8% using the 0.1 ml/min sample line at 30 samples/h and was therefore not included in the calculations. Units were defined as the amount of enzyme required to prevent the reduction of 1 μmol of DCIP/min at 25°C, pH 7.8. The formula used to convert the absorbance peak heights to enzyme activity concentrations was U/ml = $2.312 \times 10^{-3} \times$ absorbance peak height. For convenience of presentation, the enzyme activities have been expressed in milliunits (10^{-3} units, mU), unless otherwise noted.

Assay of GSH concentration after enzyme reaction. In order to determine the effect of the enzyme concentration on the concentration of GSH remaining after the enzyme reactor, the DCIP reagent was replaced with 2 mM DTNB (Sigma) in 0.2 M Tris·HCl, pH 8.2, and the absorbance was measured at 420 nm. The system was calibrated with GSH solutions of known concentration with no enzyme present, to construct a linear standard curve of absorbance at 420 nm vs GSH concentration. This reaction was not used to monitor the enzyme activity because of the nonlinear relationship between the enzyme activity and the absorbance at 420 nm (11,12).

Manual coupled GSH-Px assays. GSH-Px was measured by the method of Paglia and Valentine (5) as modified by Tappel (18). The conditions were 0.25 mM GSH, 30 μg/ml CHPO, 0.12 mM NADPH, 1 U/ml GSH reductase, 8.7 mM NaCN, 50 mM Tris·HCl, 0.1 mM EDTA, pH 7.8, 37°C. The units in this assay are defined as the amount of enzyme which causes the oxidation of 1 μmol of NADPH to $NADP^+$ per minute. One unit in the coupled assay was equivalent to 167.5 units based on DCIP.

Specific inhibition of selenium-dependent GSH-Px by iodoacetic acid. Twenty microliters of 40 mM GSH was added to 0.4 ml of each sample and the samples were incubated for 15 min at 37°C to reduce the enzyme. Each sample was split into two aliquots of 0.19 ml each and 10 μl of either distilled water or 0.1 M iodoacetic acid was added to each. The control and inhibited aliquots were incubated for 15 min at 37°C, then for 15 min at room temperature. To each aliquot, 3.42 ml of 50 mM Tris·HCl/0.1 mM EDTA/0.5% Triton X-100, pH 7.8, was

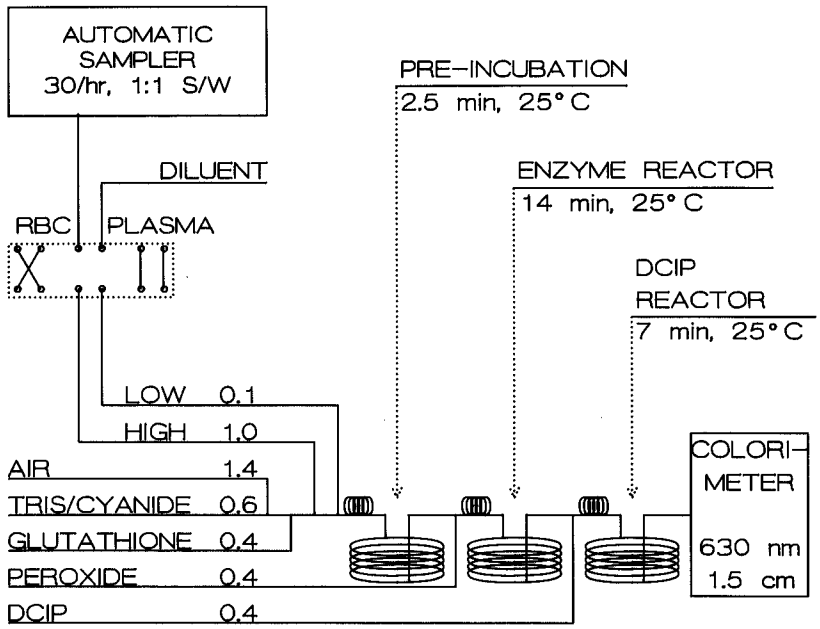

FIG. 1. Schematic diagram of continuous-flow analytical system. Numbers over the reagent lines represent the nominal flow rates of the pump tubes in ml/min. The different connections of the sampling and diluent lines for assays of erythrocyte or plasma samples are shown within the dotted box. RBC, erythrocytes; diluent, 50 mM Tris·HCl/0.1 mM EDTA/0.5% Triton X-100, pH 7.8.

added to dilute the enzyme for assay. For each set of samples another control containing iodoacetic acid but no enzyme was also prepared and assayed along with the enzyme samples. The peak heights for the iodoacetic acid containing enzyme samples were corrected for the small baseline shift caused by the presence of iodoacetic acid.

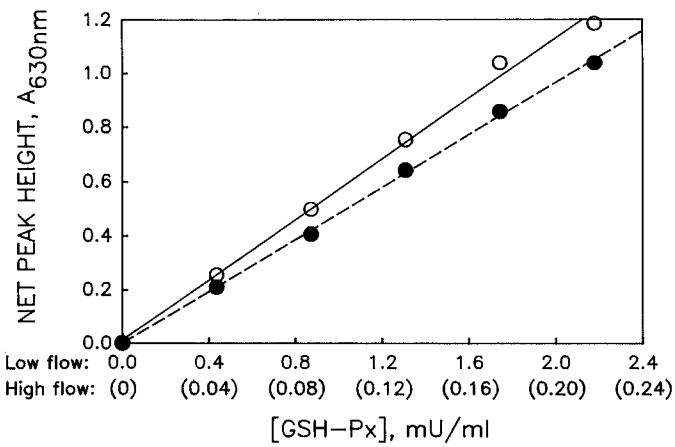

FIG. 2. Standard calibration graphs. Bovine erythrocyte GSH-Px standards (Sigma No. G-6137) were assayed with CHPO as the oxidizing substrate using the high flow sampling line (1.0 ml/min, solid circles) or using the low flow sampling line (0.1 ml/min, open circles). The lower scale labels on the horizontal axis (in parentheses) represent the enzyme concentrations used with the high flow sampling line and the upper labels represent the enzyme concentrations used with the low flow sampling line.

RESULTS

Optimization of reaction conditions. The concentrations of GSH and CHPO were chosen to minimize interference from heme proteins, whose activities became significant at higher substrate concentrations. The NaCN concentration and the enzyme reaction pH were adjusted to give the best selectivity for GSH-Px with respect to heme proteins. Sodium azide, potassium fluoride, EDTA, and hydroxylamine hydrochloride were not as effective as cyanide in this regard. The reaction was conducted at room temperature to minimize heme protein interferences. To compensate for the lower enzyme reaction temperature, the length of the enzyme reaction was increased until sufficient sensitivity was attained. Because the reaction between DCIP and GSH was incomplete (excess GSH and partial reduction of DCIP), the length of the reaction coil in the "DCIP reactor" (Fig. 1) was not critical, although it affected the optimum ratio of DCIP to GSH. This optimum ratio was determined by systematically varying the DCIP concentration while holding the GSH and CHPO concentrations constant. Increasing the DCIP increased the sensitivity of the assay (slope of the standard curve) but also increased the baseline absorbance. A concentration of 14 absorbance units/ml at 624 nm (0.68 mM) gave adequate sensitivity while keeping the baseline below 0.3 absorbance unit and free of excessive noise.

Linearity of response. The chemistry of the enzyme reaction is represented by

TABLE 1

Within-Day and Between-Day Precision of the Automated GSH-Px Assay with DCIP[a]

Sample type	Mean activity (mU/ml)	Within-day standard deviation		Pure between-day standard deviation[b]	
		(mU/ml)	(%RSD)[c]	(mU/ml)	(%RSD)[c]
Low sensitivity (0.1 ml/min sample flow rate)					
GSH-Px	0.356	0.0043	1.2	0.0254	7.1
GSH-Px	1.112	0.0233	2.1	0.0327	2.9
Human erythrocytes, 1:40	0.475	0.0102	2.2	0.0174	3.6
High sensitivity (1.0 ml/min sample flow rate)					
GSH-Px	0.0362	0.0009	2.5	0.0009	2.7
GSH-Px	0.1540	0.0017	1.1	0.0020	1.3
Human plasma, 1:20	0.0259	0.0017[d]	6.4[d]	0.0017[d]	6.4[d]

[a] Sets of four replicates were assayed on 4 separate days. Variances were separated and estimated by one-way analysis of variance (19).
[b] "Pure" between-day standard deviation does not include the contribution from within-day sources of variance (20).
[c] Relative standard deviation: (SD/mean) × 100%.
[d] Analysis of variance showed no significant differences between within-day variance and between-day variance.

$$ROOH + 2GSH \rightarrow ROH + GSSG + H_2O, \quad [1]$$

and the indicator reaction is

$$\text{DCIP–quinone (blue)} + 2GSH \rightarrow \text{DCIP–alcohol (clear)} + GSSG. \quad [2]$$

The depletion of GSH during the enzyme reaction [1] is detected by its effect on the rate of the indicator reaction [2]. When there is no enzyme present, the GSH concentration is not decreased by reaction [1] and the blue color of the DCIP is subsequently bleached to baseline levels in reaction [2]. When enzyme is present, some of the GSH is oxidized to GSSG in reaction [1] causing the rate of reaction [2] to decrease, which results in the appearance of blue color due to DCIP–quinone that is not reduced in reaction [2].

The concentration of GSH remaining after the enzyme reaction [2] was determined as described under Materials and Methods after reaction with graded levels of GSH-Px. The final GSH concentration was found to have a linear dependence on the reciprocal of the enzyme concentration over at least a fourfold range of enzyme concentrations ($r = 0.9991$, $n = 5$), represented by

$$[GSH]_{final} = \text{constant} \times 1/[\text{GSH-Px}]. \quad [3]$$

Equation [3] reflects the fact that higher enzyme concentrations result in lower GSH concentrations after a given reaction time.

To study the dependence of the residual DCIP absorbance on the GSH concentration, the flow system was run with graded levels of GSH in the absence of enzyme. The residual DCIP absorbance was found to have a linear dependence on the reciprocal of the GSH concentration over at least a fivefold range of GSH concentrations ($r = 0.9986$, $n = 8$), represented by

$$[DCIP]_{residual} = \text{constant} \times 1/[GSH]_{final}. \quad [4]$$

Equation [4] reflects the fact that the DCIP color is bleached faster and more completely as the GSH concentration increases. Substituting Eq. [3] for $[GSH]_{final}$ in Eq. [4] gives an overall equation of

$$[DCIP]_{residual} = \text{constant} \times [\text{GSH-Px}], \quad [5]$$

thus rationalizing the observed linear dependence of absorbance peak height on GSH-Px concentration. Figure 2 shows the response of peak height to increasing concentrations of GSH-Px, using either the 1.0 ml/min sampling line or the 0.1 ml/min sampling line. The response was linear from 0 to 0.22 mU/ml at 1.0 ml/min and from 0 to 2.2 mU/ml at 0.1 ml/min with correlation coefficients greater than 0.99.

Repeatability and reproducibility. To estimate the within-day repeatability and the between-day reproducibility, four samples of each sample type were analyzed on each of 4 separate days. A one-way analysis of variance was used to separate the total variance into contributions from within-day sources and between-day sources as recommended by Miller and Miller (19). Using this approach, the between-day variance is an estimate of the "pure between-day variance" and does not include the within-day variance (20). The separated variances were used to calculate the standard deviation data shown in Table 1.

There was no significant difference between the precision with the high sample flow rate and the precision with the low sample flow rate. The precision with plasma

TABLE 2

Selectivity of the Automated GSH-Px Assay with DCIP

	GSH-Px activity: percentage of control							
	CHPO				H_2O_2			
	−NaCN[b]		+NaCN[b]		−NaCN[b]		+NaCN[b]	
Enzyme[a]	−IAA[c]	+IAA[c]	−IAA[c]	+IAA[c]	−IAA[c]	+IAA[c]	−IAA[c]	+IAA[c]
GSH-Px (Sigma)	100	0.8	49.4	1.0	92.2	0.2	47.0	1.5
Erythrocytes	100	42.4	35.1	3.6	Neg[d]	Neg[d]	38.9	12.1
Plasma	100	49.4	134.8	56.2	102.2	59.0	103.4	59.0
Hemoglobin	100	103.5	42.4	43.5	62.1	57.4	53.2	41.4
Myoglobin	100	128.0	14.3	19.4	113.9	128.7	22.4	25.4
Cytochrome *c*	100	105.8	7.1	9.5	45.8	52.4	7.6	10.9
Bovine GST[e]	100	118.3	44.2	61.4	N.D.[f]	1.6	N.D.[f]	2.4
Rat GST[e]	100	106.1	45.7	56.9	8.1	2.0	8.7	2.6
Equine GST[e]	100	163.9	131.6	178.2	N.D.[f]	11.4	N.D.[f]	12.7

[a] Enzyme sources and concentrations were bovine erythrocyte GSH-Px, 0.029 mU/ml; human erythrocytes, 1:120 dilution; human plasma, 1:20 dilution; bovine erythrocyte hemoglobin, 3 mg/ml; horse heart myoglobin, 1.5 mg/ml; horse heart cytochrome *c*, 3 mg/ml; bovine liver GSH *S*-transferase, 0.4 U/ml; rat liver GSH *S*-transferase, 0.74 U/ml; horse liver GSH *S*-transferase, 1.3 U/ml. GSH *S*-transferase units were micromoles of 1-chloro-2, 4-dinitrobenzene conjugated per minute with 2.5 mM GSH, pH 6.5, 25°C.

[b] Assays were conducted with (+NaCN) or without (−NaCN) 8.7 mM NaCN in the reaction mixture.

[c] Enzyme samples were assayed after pretreatment with iodoacetic acid (+IAA) or without pretreatment (−IAA) as described under Materials and Methods.

[d] Negative peaks, below baseline.

[e] GSH *S*-transferase.

[f] Not detectable.

was not as good as that with erythrocytes, probably because of the much lower enzyme activity in plasma. The limit of detection was estimated as three times the standard deviation of the lowest standard tested, or 2.7×10^{-6} U/ml.

Accuracy of the method. The accuracy of the method was evaluated by comparison to the manual coupled assay in a variety of plasma/serum and erythrocyte samples from rats, rabbits, monkeys, and humans, using CHPO as the oxidizing substrate in both assay methods. Since the typical enzyme levels in plasma/serum are 10 to 100-fold lower than those in erythrocytes, accuracy was evaluated separately for the two sample types by linear regression of the automated assay values on the manual coupled assay values after converting the coupled assay units to DCIP units.

Very close agreement was found between the two methods in erythrocyte samples, with $r = 0.997$, slope $= 0.976 \pm 0.040$ (±SE), intercept $= -0.170 \pm 0.261$ (±SE), and $n = 8$ (range: 3.63 to 87.2 mU/ml). The agreement in plasma/serum samples was not as good as that in erythrocytes, with $r = 0.923$, slope $= 0.938 \pm 0.090$ (±SE), intercept $= -0.0006 \pm 0.033$ (±SE), and $n = 19$ (range: 0.36 to 2.18 mU/ml). The poorer correlation with plasma/serum samples is at least partially due to the very low activity and the correspondingly greater imprecision of both assay methods in plasma/serum samples (Table 1). These results demonstrate an absence of significant constant or proportional errors between the two methods at the 95% confidence level.

Selectivity and interferences. Iodoacetic acid was used as a specific inhibitor of selenium-dependent GSH-Px activity. Standards, human erythrocytes, human plasma, and test proteins were assayed with and without iodoacetic acid treatment, with and without the presence of cyanide in the buffer, and with CHPO or H_2O_2 as oxidizing substrate (Table 2). Bovine erythrocyte GSH-Px was inhibited about one-half by cyanide and 97 to 99% by iodoacetic acid, with either CHPO or H_2O_2 as substrate. Although cyanide caused a loss of half the sensitivity for standard enzyme, it was very effective at decreasing the iodoacetic acid-insensitive activity present in erythrocytes. With CHPO as substrate and in the presence of cyanide, the iodoacetic acid-sensitive activity accounted for 90% of the total erythrocyte activity. With H_2O_2 as substrate, the interference from erythrocytes caused negative peaks when cyanide was omitted. When H_2O_2 was used as substrate in the presence of cyanide, the iodoacetic acid-sensitive activity accounted for 69% of the total erythrocyte activity, showing that cyanide was only partially effective at removing the strong interference of erythrocytes on assays with H_2O_2.

The activity in plasma was increased by the presence of cyanide when CHPO was substrate and was only partially inhibited by iodoacetic acid with either substrate, with or without cyanide. It could not be determined from

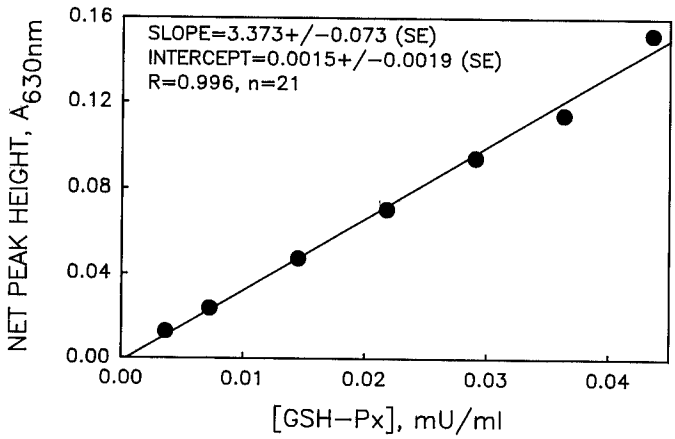

FIG. 3. Linear response to low GSH-Px concentrations. The system was run with the standard conditions, using the 1.0 ml/min sampling line and with the colorimeter sensitivity increased eightfold (0.25 AUFS). Three replicates were analyzed at each level of bovine erythrocyte GSH-Px. Only the means of the replicates are plotted with symbols, although all of the data points were used for calculation of the regression line. Tests for linearity included outlier residuals (19), Wald-Wolfowitz runs test of residuals (19), and *F* test for comparison of the sample variance to the regression variance (24) and showed no evidence of significant deviations from linearity at the 95% confidence level.

these data whether plasma contained an interfering activity or if these results were due to the many reported differences between the plasma and the cellular enzymes (21). In any event, the lack of significant bias between the automated assay and the manual coupled assay in plasma/serum samples suggests that any interferences from plasma/serum are common to both assay methods.

The apparent GSH-Px activities of the heme proteins were inhibited by cyanide, in the relative order of cytochrome *c* > myoglobin > hemoglobin. Although hemoglobin was the most resistant to inhibition by cyanide, the fact that the erythrocyte activity was 90% iodoacetic acid-sensitive when CHPO was substrate in the presence of cyanide indicates that the selectivity with erythrocyte samples under these conditions was adequate. The non-selenium-dependent GSH-Px activities of the GSH *S*-transferases were not selectively inhibited by cyanide, but their activities were dramatically decreased when H_2O_2 was substrate, as has been reported for the manual coupled assay (22).

The selectivity and susceptibility to interferences are essentially the same as those for the standard coupled assay. The automated assay with H_2O_2 is highly selective against the non-selenium-dependent activity of GSH *S*-transferases and the automated assay with CHPO measures both the non-selenium-dependent and the selenium-dependent activities, but is selective against interference from heme proteins.

DISCUSSION

This assay is suitable for routine determinations of GSH-Px activity in blood samples and especially for high volume applications where throughput and cost are major concerns. At a sampling rate of 30/h, the sample-to-sample carryover is less than 0.8%. Higher throughputs could be obtained with faster sampling speeds at the cost of increased carryover or decreased sensitivity. Newer technology, such as "micro-continuous-flow" analyzers should allow higher throughput and lower reagent cost without sacrificing sensitivity or increasing carryover. This method may not be suitable for some applications in enzyme kinetics because the concentration of GSH affects the rate of the DCIP indicator reaction as well as the enzyme reaction. A manual version of this assay may not be practical because of the need for precise timing of the absorbance readings in the DCIP indicator reaction step.

While it may at first seem surprising that the net effect of reactions [1] and [2] is to give a linear response to GSH-Px concentration, it could be predicted based on the dependence of the rates of reactions [1] and [2] upon the GSH concentration. The GSH-Px reaction [1] is first order with respect to GSH (9) and reaction [2] is expected to be first order with respect to GSH also. Since the dependence of reaction [2] upon the rate of reaction [1] is opposite to the dependence of reaction [1] upon GSH-Px, that is, when the rate of reaction [1] is at its minimum (GSH-Px = 0) the rate of reaction [2] is at its maximum (blue color = baseline) and vice versa, the changes in the rates of reactions [1] and [2] due to the changing GSH concentration would be expected to cancel each other out, as is in fact observed. The continuation of the GSH-Px reaction [1] in the DCIP reactor apparently does not affect this relationship since we were able to establish a linear response after independently varying the temperatures of the enzyme and indicator reactions between 25 and 37°C and varying the time in the enzyme reactor from 0 to 21 min and the time in the indicator reactor from 3 to 7 min. Using the conditions described under Materials and Methods, we were able to demonstrate that more than 80% of the enzyme reaction occurs in the enzyme reactor by comparing the peak heights with and without the enzyme reactor in the system. This is due to the twofold longer enzyme reaction time and the fact that the rate of the enzyme reaction decreases as the GSH is consumed in reaction [1] and drops precipitously when the DCIP indicator reaction [2] starts to consume the remaining GSH.

The main determinants of the linear response to GSH-Px are "balancing" of the GSH and DCIP concentrations and maintenance of sufficiently high CHPO concentrations relative to GSH and DCIP. Linearity at the lower GSH-Px concentrations depends on the ratio of GSH to DCIP being such that most, but not all, of the DCIP is bleached by GSH in the absence of enzyme. We found baseline absorbances of 0.05 to 0.3 to be suitable. Figure 3 shows that linearity is maintained all the way down to near the detection limit using our standard conditions. Linearity at the higher GSH-Px concentrations

depends on the CHPO concentration being sufficiently large relative to the GSH and DCIP. In general, higher GSH and DCIP concentrations require higher CHPO concentrations to maintain linearity. Increasing the CHPO concentration while holding GSH and DCIP constant extends the linear range to higher GSH-Px concentrations. The top of the linear range is, of course, limited by the DCIP concentration, which can only produce a finite amount of color. We were able to establish a linear response to GSH-Px over the following reactant concentration ranges: GSH, 0.25 to 2.3 mM; DCIP, 0.1 to 0.8 mM; and CHPO, 0.125 to 20 mM.

The sensitivity of the assay to GSH-Px is increased as the reactant concentrations are increased. However, the interfering heme-catalyzed reactions are increased even more, especially by CHPO. We found the conditions described under Materials and Methods to be an adequate compromise between sensitivity and selectivity for assays in erythrocytes. A system dedicated to assays in plasma or serum could be made much more sensitive to GSH-Px by increasing the reactant concentrations and the temperature and pH of the enzyme reaction.

The enzyme activity unit definition used in this work, micromoles of DCIP "spared" per minute, is dependent on the reactant concentrations, as well as the time and temperature of the indicator reaction. This is because the reaction between GSH and DCIP is incomplete and the bleaching of DCIP is readily reversed upon standing. Therefore, each new flow system should be calibrated against a published assay method, as we have done here.

The lack of a standard enzyme unit definition has hampered interlaboratory comparisons of GSH-Px activities for many years (23). This situation has arisen because of the kinetics of GSH-Px which cannot be simultaneously saturated with respect to both GSH and hydroperoxide (9), causing the observed activity to be dependent on the substrate concentrations. Even the standard coupled assay is affected by the kinetics as well as by the pH, the temperature, and the presence of inhibitors (e.g., cyanide or azide). Therefore, there are several widely used versions of the "standard" coupled assay, which all yield different activities. Since there is no widely accepted unit definition for GSH-Px anyway, there is no disadvantage in our choice of units. Under the conditions described under Materials and Methods, one enzyme activity unit in the coupled assay is equivalent to 167.5 units based on DCIP. The best solution to this long-standing problem would be the use of a common calibration or reference standard by all laboratories, similar to our use of the Sigma enzyme as an interassay calibration standard between the coupled assay and our method.

The data presented here demonstrate that monitoring of GSH oxidation with DCIP can be used for the sensitive and selective determination of GSH-Px activity in blood of several species. Enzyme activities determined by this method may be compared directly to activities determined by the coupled assay as long as a common reference standard is used to calibrate both assays. The automated method has the advantages of less required labor, higher throughput, and lower materials cost than the coupled assay, without sacrificing sensitivity, accuracy, or precision.

REFERENCES

1. Mills, G. C. (1957) *J. Biol. Chem.* **229,** 189–197.
2. Cohen, G., and Hochstein, P. (1963) *Biochemistry* **2,** 1420–1428.
3. Rotruck, J. T., Pope, A. L., Ganther, H. E., Swanson, A. B., Hafeman, D. G., and Hoekstra, W. G. (1973) *Science* **179,** 588–590.
4. Forstrom, J. W., Zakowski, J. J., and Tappel, A. L. (1978) *Biochemistry* **17,** 2639–2644.
5. Paglia, D. E., and Valentine, W. N. (1967) *J. Lab. Clin. Med.* **70,** 158–169.
6. Little, C., and O'Brien, P. J. (1968) *Biochem. Biophys. Res. Commun.* **31,** 145–150.
7. Nakamura, W., Hosoda, S., and Hayashi, K. (1974) *Biochim. Biophys. Acta* **358,** 251–261.
8. Thomson, C. D., Rea, H. M., Doesburg, V. M., and Robinson, M. F. (1977) *Brit. J. Nutr.* **37,** 457–460.
9. Forstrom, J. W., Stults, F. H., and Tappel, A. L. (1979) *Arch. Biochem. Biophys.* **193,** 51–55.
10. Splittgerber, A. G., and Tappel, A. L. (1979) *Arch. Biochem. Biophys.* **197,** 534–542.
11. Gross, R. T., Bracci, R., Rudolph, N., Schroeder, E., and Kochen, J. A. (1967) *Blood* **29,** 481–493.
12. Hafeman, D. G., Sunde, R. A., and Hoekstra, W. G. (1974) *J. Nutr.* **104,** 580–587.
13. Faraji, B., Kang, H. K., and Valentine, J. L. (1987) *Clin. Chem.* **33,** 539–543.
14. Zakowski, J. J., and Tappel, A. L. (1978) *Anal. Biochem.* **89,** 430–436.
15. Xia, Q., Li, L., Wu, D., and Xu, G. (1987) *Chromatogram* **8**(3), 11–12.
16. Jaskot, R. H., Charlet, E. G., Grose, E. C., Grady, M. A., and Roycroft, J. H. (1983) *J. Anal. Toxicol.* **7,** 86–88.
17. Jacobson, B., Quigley, G., and Lockitch, G. (1988) *Clin. Chem.* **34,** 2164–2165.
18. Tappel, A. L. (1978) *in* Methods in Enzymology (Fleischer, S., and Packer, L., Eds.), Vol. 52, Part C, pp. 506–513, Academic Press, New York.
19. Miller, J. C., and Miller, J. N. (1984) Statistics for Analytical Chemistry, pp. 73–124, Ellis Horwood, Ltd., Chichester.
20. Bookbinder, M. J., and Panosian, K. J. (1986) *Clin. Chem.* **32,** 1734–1737.
21. Takahashi, K., Avissar, N., Whitin, J., and Cohen, H. (1987) *Arch. Biochem. Biophys.* **256,** 677–686.
22. Lawrence, R. A., and Burk, R. F. (1976) *Biochem. Biophys. Res. Commun.* **71,** 952–958.
23. Levander, O. A. (1985) *Fed. Proc.* **44,** 2579–2583.
24. Taylor, J. K. (1987) Quality Assurance of Chemical Measurements, pp. 101–103, Lewis Publishers, Inc., Chelsea, MI.